Use of Butanol in the Purification of the Alkaline Phosphatase of Bovine Milk

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Introduction

Preparations of alkaline phosphatase of bovine milk contain a lipide-like substance difficult to remove (1,2). Recently the use of *n*-butanol for the dissociation of lipide–protein complexes was described (3). Application of this technique to skim milk followed by acetone fractionation has given by a simple procedure more than a 1000-fold increase in purity of the phosphatase, with good yields. Butanol has been used for the purification of the alkaline phosphatase in cream (3), but application of the method to skim milk also was considered desirable because of its availability and difference in composition. The success of the butanol method with skim milk is further evidence of the general applicability of the method.

Method

The assay of the phosphatase and the unit of activity have been described (1,2).

Protein concentrations were determined initially from the N content and the factor 6.25; they are determined routinely with the Biuret reagent on acid precipitates. The protein is precipitated with 0.1 ml. of 1.0 *M* sodium tungstate and 3.0 ml. of 10% trichloroacetic acid to each 1.0 ml. of protein solution. Without the tungstate the protein fractions most soluble in acetone are incompletely (about 50%) precipitated with 10% trichloroacetic acid. Ultraviolet absorption as a measure of protein concentration was investigated, but as much as a four-fold variation in the absorption per unit of protein for the different fractions was too great to make the method useful.

The phosphatase was prepared from unpasteurized skim milk. Commercial skim milk, purchased in 60-l. lots, has consistently had phosphatase activities close to that shown in Table I. In this table, the extent of purification of the phosphatase in the various fractions is tabulated. The casein is removed from the skim milk by clotting

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with rennet and filtering through a muslin bag. Solid ammonium sulfate is added to the whey filtrate until the concentration is 2.3 *M*. Nine grams of the wet precipitate is dissolved in water to a volume of 100 ml.; the pH is 6.0, the ammonium sulfate concentration 0.15 *M*, and the protein concentration about 2.0%. Sixty-seven ml. of *n*-butanol is added for each 100 ml. of solution, and the mixture is stirred for 5 min. at 37°. The mixture is centrifuged, and the sublevel aqueous fluid is drawn off. The butanol gel is stirred with 25 ml. of water, and the aqueous portion recovered as before. The two aqueous solutions are combined. For each 100 ml., 60 ml. of acetone is added, and the mixture is stirred at 37° for 5 min. The precipitate, which contains no phosphatase, is removed. The protein in the supernatant fluid is precipitated with 114 ml. of acetone for each 100 ml. This precipitate, which contains the phosphatase, is dissolved in about 15 ml. of 0.1 *M* tris(hydroxymethyl)aminomethane–HCl (MAM) buffer, pH 8.0. The protein concentration is about 2.5%. The distribution of protein in this preliminary fractionation is approximately as follows: butanol gel, 45%; first

TABLE I

Purification of Alkaline Phosphatase

The phosphatase activities given are subject to a variation of ±10% depending largely on the specific activity of the starting material.

Fraction	No.	Specific activity *units/1 mg.*	Yield over-all %
Skim milk	1	8 (250 units/1 ml.)	100
Whey	2	20	60
Ammonium sulfate precipitate, 2.3 *M*	3	45	60
After butanol treatment	4	80	57
First acetone fraction	5	110	57
Second acetone fraction	6	2,000–5,000	52
Filter-Cel filtrate	7	4,000–10,000	47

acetone precipitate, 20%; phosphatase fraction (No. 5), 35%. The purification effect by the butanol step alone is small (less than two-fold), but without this treatment, subsequent fractionation is impossible.

In the next step, acetone is added to the phosphatase solution (No. 5) until a definite precipitate is obtained. Approximately 8.0 ml. of acetone is required per 10.0 ml. of solution. The precipitate from 10 ml. of solution is dissolved in 10.0 ml. of MAM buffer; the protein concentration is 0.06–0.15%; activity is 2000–5000 units/1 mg. This step should be done in 10-ml. trial portions to determine the conditions for obtaining all the phosphatase with the minimal amount of protein, since, with care, a greater increase in purity can be obtained at this point than in any single subsequent step that has been devised. The bulk of the protein, the nonphosphatase portion, is precipitated by increasing the amount of acetone to 2 vol.

In the final step, 5 g. of Filter-Cel is added per 100 ml. of the phosphatase solution, and the mixture is filtered through a Büchner funnel. Not only are nearly water-clear solutions obtained by this procedure, but as much as 50% of the nonphosphatase protein is adsorbed on the Filter-Cel, with a two-fold increase in purity of the phosphatase. The best preparations of the phosphatase at this stage are more than 1000-fold purer than found in skim milk. With repetition of the Filter-Cel filtration and precipitation with acetone, preparations five times this level of purity have been obtained. An electrophoretic examination of a 0.27% solution of the phosphatase containing 41,700 units/1 mg. showed that at least three components were present.

Phosphodiesterase [nucleophosphatase (4)] activity in milk whey cannot be demonstrated with a manometric procedure (5) in which ribonucleic acid is used as the substrate. The most highly purified phosphatase preparations, however, contained 0.45–3.7 units of diesterase/10 mg. The activity did not parallel the phosphatase activity; the variation was as much as three-fold. Thus it appears that there is phosphodiesterase in cow's milk and that it is concentrated in the course of purifying the alkaline phosphatase. Alkaline phosphatase preparations from calf intestinal mucosa hydrolyze diesters of phosphoric acid also. At one time it was thought this might be a property of the alkaline phosphatase (phosphomonoesterase) (6). However, a comparison of the ratio of activities in different crude preparations and fractionation studies wherein the activity ratios differed 10-fold led to the conclusion that they must be different enzymes (7).

Discussion

Morton (3), using the butanol treatment with buttermilk from cream of 60–70% butterfat as the starting material, obtained a 5000-fold purification of the phosphatase, as compared with milk. The preparation of this material, other than the use of butanol, was not described. The preparations obtained in the present study of this level of purity contained three components. With the removal of the lipide, however, it is expected that the phosphatase will be amenable to further purification. The stability in the presence of acetone indicates that organic solvents will be useful fractionating agents.

The importance of *n*-butanol for purification of milk phosphatase is emphasized by the lack of success with the many other reagents tried. Since the phosphatase is associated with a lipide-like component, various organic solvents (ethyl ether, acetone, chloroform, toluene) were tried, both on aqueous solutions and ammonium sulfate precipitates. The phosphatase was not inactivated, but it could not be obtained in true solution, and in some cases insoluble fractions were obtained that were not amenable to further fractionation. The striking success of *n*-butanol, as compared with other organic solvents, in the purification of phosphatase in skim milk lends weight to Morton's belief (3) that

n-butanol is specific in its ability to dissociate lipide–protein complexes or even protein–protein complexes.

The enzyme lecithinase was used on the whey phosphatase without success. Trypsin, which successfully brings into solution the alkaline phosphatase in calf intestinal mucosa (5), was only partially successful with milk. In an early paper (1) a 60-fold purification and removal of the lipide fraction was reported. Subsequent studies (2) showed that a lipide-like fraction was still present and that it was characterized by adsorption on Filter-Cel from salt-containing solutions. A study of the phosphatase in a solution of the "2.3 *M* ammonium sulfate precipitate" under conditions for maximal adsorption ($\lesssim$600 units/1 ml., pH 8.0, 1.5 *M* NaCl, 0.4 g. Filter-Cel/10 ml.) (2) showed that initially only about 20% of the phosphatase complex was adsorbed on Filter-Cel. However, when this phosphatase was treated with trypsin at pH 8.5 for 18 hr., the phosphatase adsorption increased to about 70%. This change has not been analyzed in detail, but it has been found that a considerable increase in adsorption occurs after the solution has been at pH 8.5 for 18 hr. without trypsin. The phosphatase that can be adsorbed on Filter-Cel has been obtained in moderate purity (7500 units/1 mg.), but the solutions are opalescent even at a concentration of 0.2% protein. Phosphatase prepared by the butanol method is not adsorbed on Filter-Cel nor is there any adsorption after treatment with trypsin at pH 8.5.

Prior to the use of butanol, the highest purity of nonadsorbed phosphatase was obtained with lipase treatment (2). Unfortunately, only about 20% of the phosphatase was obtained in true solution, and the rest was on an insoluble fraction that could not be further fractionated. However, the effectiveness of lipase is further evidence of the presence of lipide in the phosphatase complex.

Summary

The alkaline phosphatase of bovine milk has been purified more than 1000-fold by dissociation of the phosphatase complex with *n*-butanol and subsequent fractionation with acetone. This extends the usefulness of the *n*-butanol treatment originally used for the purification of the alkaline phosphatase in cream.

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